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BTB/POZ-Zinc Finger Protein Abrupt Suppresses Dendritic Branching in a Neuronal Subtype-Specific and Dosage-Dependent Manner

Wenjun Li,[1,2,3] Fay Wang,[1,2,3] Laurent Menut,[1] and Fen-Biao Gao[1,2,3,*]
[1]Gladstone Institute of Neurological Disease
[2]Program in Neuroscience
[3]Department of Neurology
University of California, San Francisco
San Francisco, California 94141

Summary

How dendrites of different neuronal subtypes exhibit distinct branching patterns during development remains largely unknown. Here we report the mapping and identification of loss-of-function mutations in the *abrupt* (*ab*) gene that increased the number of dendritic branches of multiple dendritic (MD) sensory neurons in *Drosophila* embryos. *Ab* encodes an evolutionarily conserved transcription factor that contains a BTB/POZ domain and C_2H_2 zinc finger motifs. We show that *ab* has a cell-autonomous function in postmitotic neurons to limit dendritic branching. Ab and the homeodomain protein Cut are expressed in distinct but complementary subsets of MD neurons, and Ab functions in a transcriptional program that does not require Cut. Deleting one copy of *ab* or overexpressing *ab* had opposite effects on the formation of higher-order dendritic branches, suggesting that the Ab level in a specific neuron directly regulates dendritic complexity. These results demonstrate that dendritic branching can be suppressed by neuronal subtype-specific transcription factors in a cell-autonomous and dosage-dependent manner.

Introduction

The development of dendritic branching patterns specific to various neuronal subtypes is critical for the proper function of the nervous system (Ramón y Cajal, 1911; Masland, 2001). It is largely unknown how the shapes of different neurons are specified during development or what are the relative contributions of intrinsic factors and extracellular cues to this important process. When dissociated from tissues and cultured at low densities, many neurons develop axonal and dendritic morphologies that are characteristic of the same subtype of neurons in vivo, suggesting that an intrinsic genetic program controls neuronal morphogenesis (reviewed in Bradke and Dotti, 2000; Craig and Banker, 1994). It remains a major challenge to identify the key molecular players in this intrinsic program and to understand at the mechanistic level how they control dendrite morphogenesis during normal development (reviewed in Cline, 2001; Gao and Bogert, 2003; Jan and Jan, 2003; McAllister, 2000; Miller and Kaplan, 2003; Scott and Luo, 2001; Wong and Ghosh, 2002).

Studies during the last decade or so have identified and characterized several classes of intracellular molecules that are important in dendritic development. These molecules include actin cytoskeleton regulatory proteins (e.g., recently Li and Gao, 2003; Martinez, et al., 2003; Penzes et al., 2003; Yu and Malenka, 2003), RNA binding proteins (e.g., Eom et al., 2003; Lee et al., 2003; Ye et al., 2004), and transcription factors (e.g., Aizawa et al., 2004; Gaudillière et al., 2004; Grueber et al., 2003; Komiyama et al., 2003).

Transcription factors have well-documented roles in the specification of neuronal cell fate (Bertrand et al., 2002; Jessell, 2000). Since dendritic morphology is an important aspect of neuronal identity, some proteins that function in precursor cells to control neuronal fate are likely to have a direct or indirect role in regulating dendritic morphogenesis. For instance, Sequoia, a Tramtrack-related zinc finger protein that is exclusively expressed in the developing *Drosophila* nervous system, plays a role in both cell fate determination and dendritic growth (Brenman et al., 2001). Hamlet, another zinc finger nuclear protein, functions as a binary switch between an external sensory (ES) neuron (with a single dendritic branch) and a dendritic arborization (DA) neuron (with a complex dendritic arbor) (Moore et al., 2002). The homeodomain protein Cut is expressed in both precursor cells and a subset of postmitotic DA neurons and plays an important role in specifying neural identity in the peripheral nervous system (Bodmer et al., 1987; Blochlinger et al., 1988). In the absence of Cut, DA sensory neurons in *Drosophila* exhibited greatly reduced dendritic arbors (Grueber et al., 2003). Cortical neurons from *crest* (calcium-responsive transactivator) mutant mice show reduced basal but not apical dendrites (Aizawa et al., 2004). Reducing neuroD activity with a DNA template-based RNA interference method decreased the total dendritic length of granule neurons (Gaudillière et al., 2004). These studies have provided important insights into the mechanisms that normally promote dendritic growth and branching. However, transcriptional programs dedicated to inhibiting dendritic elaboration in postmitotic neurons remain to be identified.

To address this issue, we used the peripheral nervous system (PNS) of *Drosophila* embryos as a model system. This relatively simple system consists of only 44 sensory neurons in each abdominal hemisegment (Ghysen et al., 1986; Bodmer et al., 1989). Some sensory neurons elaborate extensive subepidermal dendritic arbors that can be labeled with green fluorescent protein (GFP) and easily visualized in living embryos or larvae (Bodmer and Jan, 1987; Gao et al., 1999, 2000). This system has been used to identify and characterize a number of molecules that are important in dendritic morphogenesis (reviewed in Gao and Bogert, 2003; Grueber and Jan, 2004).

Here we report that loss-of-function mutations in the *abrupt* (*ab*) gene increase the dendritic branching of MD sensory neurons in *Drosophila* embryos. *Ab* encodes an evolutionarily conserved transcription factor that is widely expressed during embryogenesis and contains a Broad, Tramtrack and Bric a brac/Poxvirus and Zinc

*Correspondence: fgao@gladstone.ucsf.edu

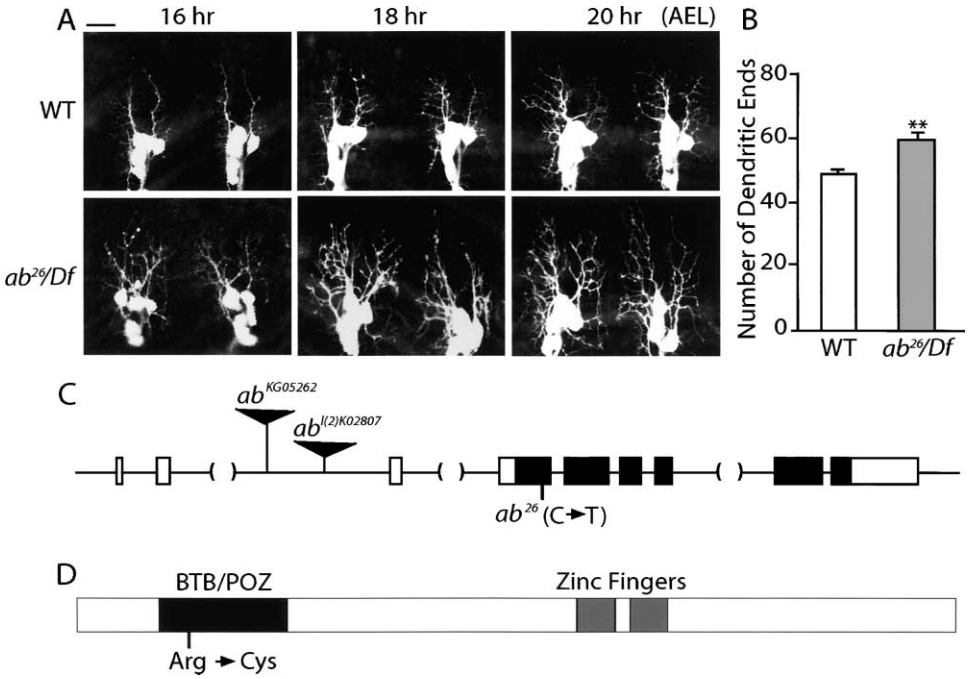

Figure 1. Identification of Loss-of-Function Mutations in *ab* that Increased Dendritic Branching of MD Neurons in *Drosophila*

(A) Dendritic branching of dorsal cluster MD neurons in wild-type (WT) or *ab* mutant embryos. *Df*: *Df(2L)FCK-20* (032D01;032F01-03, BL-5869). Images are of live embryos at 16, 18, and 20 hr AEL. The dorsal cluster MD neurons were labeled with GFP under the control of *Gal4 109(2)80*. Scale bar, 20 μm.

(B) Quantification of dendritic branching in WT and *ab* mutant embryos. Dendritic ends were counted in each dorsal cluster of the A5 segment from ten embryos in each genotype. **$p < 0.01$, by Student's t test. All values are mean ± SEM.

(C) Genomic organization of the *ab* locus. The boxes indicate exons, and the lines represent introns. Black areas indicate the coding region of the *ab* gene. The single nucleotide mutation (C to T) in the ab^{26} allele is located in the fourth exon. The two P elements [*KG05262* and *l(2)K02807*] are inserted in the second intron.

(D) Domain organization of the Ab protein. The black box indicates the BTB/POZ domain, and the two shaded boxes represent zinc finger motifs. The amino acid substitution (arginine to cysteine) in the ab^{26} allele is located in the BTB/POZ domain.

Finger (BTB/POZ) domain and C_2H_2 zinc finger motifs (Hu et al., 1995). We show that Ab is a neuronal subtype-specific intrinsic regulator whose normal function is to limit dendritic branching in postmitotic neurons, and it does so through a transcription program independent of Cut. Ab and Cut are expressed in distinct but complementary subsets of MD neurons, suggesting that transcription codes in different neurons also regulate dendritic morphology. Furthermore, we show that Ab functions in a dosage-dependent manner, suggesting that fine regulation of Ab activity in vivo could be an effective way to control dendritic branching complexity of these neurons.

Results

Identification of *ab* Mutations that Increase Dendritic Branching of MD Neurons

The dendrites of MD sensory neurons in the dorsal cluster of each abdominal hemisegment develop in a stereotypic fashion during late embryogenesis. At 16 hr after egg laying (AEL), the dorsal dendrites stop extending, and lateral branches start to extend toward adjacent segment boundaries. By 20 hr AEL, these branching patterns are relatively invariable (Gao et al., 1999; Figure 1A). With this system as an assay, a number of lethal mutations induced by ethyl methanesulfonate were isolated that resulted in abnormal dendritic development (Gao et al., 1999). One mutant, line 26, exhibited an abnormal dendritic branching pattern as early as 16 hr AEL, and homozygous mutants died at the end of embryogenesis (Figure 1A). Through complementation tests with all the available deficiency lines, we identified three lethal mutations in line 26. The mutation uncovered by *Df(2L)FCK-20* (032D01;032F01-03, BL-5869) was responsible for the dendritic phenotype and embryonic lethality. This mutation was separated from other mutations through recombination and found to be in the *ab* gene (see below). Therefore, the mutation is referred to as ab^{26} in this report.

In ab^{26}/*Df(2L)FCK-20* mutant embryos, the number of sensory neurons in the dorsal cluster remained the same as in wild-type embryos, and the MD neurons were still labeled by the MD neuron marker *Gal4 109(2)80*, suggesting that the mutation did not alter their fate (data not shown). However, the number of dendritic branches increased, and the general pattern was disorganized (Figure 1A). To quantify the difference, we counted dendritic ends of the dorsal cluster sensory neurons. ab^{26}/*Df(2L)FCK-20* mutant embryos had significantly more dendritic ends than wild-type embryos (58.6 ± 2.8 versus 48.1 ± 1.9, $n = 10$/group, $p < 0.01$) (Figure 1B).

How neurons limit their dendritic growth and branch-

ing during development is an important question. We have been interested in studying the genes whose normal functions are to suppress instead of promote dendritic growth and branching (e.g., Gao et al., 2000; Lee et al., 2003; Li and Gao, 2003). We reasoned that characterization of the nature of the mutation responsible for the dendritic phenotype in *ab*^{26}*/Df(2L)FCK-20* mutant embryos may provide additional insights into this problem. To do so, we crossed the *ab*^{26} mutant line with all the P element insertion lines in the cytological region of 032D01;032F01-03 that are available from the Bloomington Stock Center. Two lethal P element insertion lines failed to complement the lethality or, more importantly, the dendritic phenotype of the *ab*^{26} allele, suggesting that the gene affected by the P element insertion was responsible for the observed dendritic phenotype in *ab*^{26} mutants. The two P elements, *KG05262* and *l(2)K02807*, were generated and used as recessive alleles independently in two different groups, and both inserted into the second intron of *ab* (Roseman et al., 1995; Johannes and Preiss, 2002) (Figure 1C). To identify the nature of the mutation, we cloned and sequenced the *ab* locus from DNA isolated from *ab*^{26} mutant embryos. A single nucleotide mutation (C to T) in the fourth exon resulted in an arginine to cysteine substitution (Figure 1D). *Ab* encodes a transcription factor that contains a BTB/POZ domain and two C_2H_2 zinc finger motifs (Hu et al., 1995). The mutated amino acid is located in the BTB/POZ domain and is highly conserved among proteins of the BTB/POZ family. The BTB/POZ domain mediates homo- or heterodimerization with other BTB/POZ domains, which are highly conserved across species and found in hundreds of proteins (Zollman et al., 1994). The identification of the *ab*^{26} mutation in the BTB/POZ domain suggests that the domain is essential in Ab's role in controlling dendritic branching.

Ab Has a Cell-Autonomous Function in Controlling Dendritic Branching

Ab is widely expressed during embryogenesis, including CNS midline cells, a subset of muscles, and the entire epidermis (Hu et al., 1995). Although the mechanism underlying much of *ab* function is still unclear, a couple of studies indicate that *ab* is required for synaptogenesis of a subset of motoneurons and controls adult sensory cell formation and wing vein development (Hu et al., 1995; Cook et al., 2004). Ab is expressed in the nucleus of muscle cells but not motoneurons, suggesting that its effect on motoneuron axonal terminals is non-cell-autonomous (Hu et al., 1995). MD sensory neurons are sandwiched between body wall muscles and the epidermis (Bodmer and Jan, 1987). Therefore, it is not clear in which cell type Ab acts to control dendritic branching of MD neurons.

To determine whether Ab has a cell-autonomous function in MD sensory neurons to affect dendritic branching, we used the mosaic analysis with a repressible cell marker (MARCM) technique (Lee and Luo, 1999) to generate single *ab*^{26} homozygous mutant neurons in third instar larvae. In this assay system, abnormal dendritic development reflects the cell-autonomous functions of the gene of interest in postmitotic MD neurons (Lee and Luo, 1999; Sweeney et al., 2002; Grueber et al., 2002). In each dorsal cluster, there are eight MD neurons, including six DA neurons that elaborate extensive subepidermal dendritic arbors (ddaA–ddaE), one bipolar dendritic (dbd) neuron, and one tracheal innervating neuron that extends a single dendrite (Bodmer and Jan, 1987; Bodmer et al., 1989). (Please note: ddaD and ddaF neurons we described in Sweeney et al. [2002] were named as ddaF and ddaD neurons, separately, by Grueber et al. [2002]. In this study, we use the nomenclature consistent with Sweeney et al. [2002].) Wild-type dbd neurons invariably extend two unbranched dendrites along the anteroposterior axis (Figure 2A). However, dendrites of most *ab*^{26} mutant dbd neurons (3 out of 4) develop clusters of ectopic small branches (Figure 2B), a striking phenotype that we have not observed in any other dendrite mutants so far. Among the six dorsal cluster DA neurons, ddaF and ddaE neurons extend most of their dendrites toward anterior or posterior segment boundaries, respectively. Their dendritic branches are characteristically very smooth without higher-order fine processes (Figures 2C and 2F). In contrast, ddaE neurons containing the *ab*^{26} mutation have more terminal dendritic branches (Figure 2D). *Ab* mutants have significantly more dendritic ends than wild-type (101.2 ± 25.2, $n = 6$ versus 32.9 ± 1.5, $n = 17$, $p < 0.05$) (Figure 2E). A similar phenotype was observed in ddaF mutant neurons (208.3 ± 40.4, $n = 5$ versus 30.3 ± 3.3, $n = 7$, $p < 0.05$) (Figures 2F–2H). Because of the substantial increase in the number of dendritic branches, the total length of dendrites was also increased (data not shown). In these experiments, all the single ddaE or ddaF neurons observed in A1 to A7 segments were grouped together for statistic analysis. These findings suggest that *ab* has a cell-autonomous function in limiting dendritic branching. Our results here indicate that *ab*, which encodes a BTB/POZ zinc finger transcription factor (Hu et al., 1995), initiates a transcriptional program in postmitotic neurons to control dendritic complexity.

Ab Controls Dendritic Branching of a Subset of MD Neurons that Do Not Express Cut

Interestingly, the *ab*^{26} mutation affected the dendritic morphology of ddaE, ddaF, and dbd neurons (Figure 2), but not the other DA neurons in the dorsal cluster. For instance, ddaC neurons extend their highly branched dendrites to cover a large area from the anterior to the posterior segment boundaries (Figure 3A). The total number of dendritic ends was not significantly different between wild-type (616.6 ± 44.4, $n = 13$) and *ab* mutant ddaC neurons (601.4 ± 106.7, $n = 5$) (Figures 3B and 3C). Unlike ddaE and ddaF neurons, ddaA neurons had numerous spine-like fine structures on their dendritic shafts (Sweeney et al., 2002) (Figure 3D). The total number of these structures did not differ in wild-type and *ab* mutant ddaA neurons (327.9 ± 18.1, $n = 16$ versus 312.6 ± 23.9, $n = 7$) (Figures 3E and 3F). These findings suggest that Ab specifically controls dendritic branching of a subset of PNS sensory neurons—those with smooth dendritic processes and less complex branching patterns.

To further confirm that the phenotype we observed was indeed due to the cell-autonomous function of the *ab* gene, we performed a genetic rescue experiment.

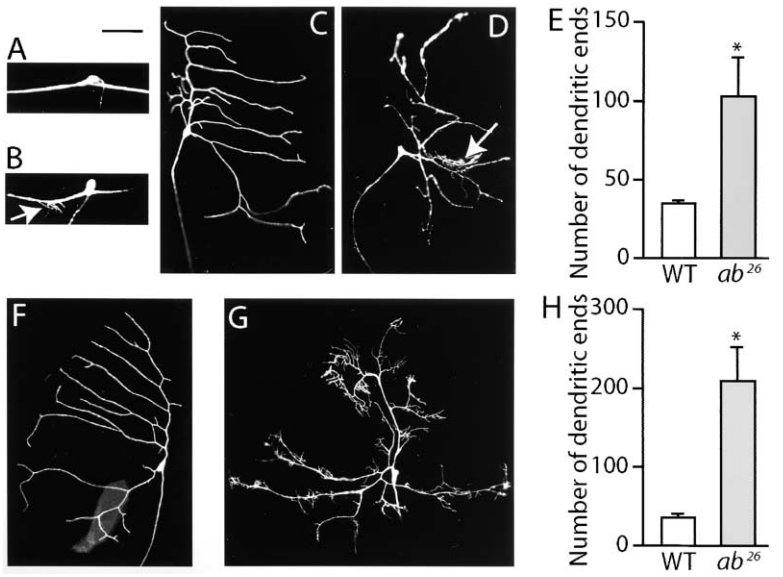

Figure 2. Ab Has a Cell-Autonomous Function in Controlling the Dendritic Branching of MD Neurons as Demonstrated by MARCM

(A) A wild-type dbd neuron. (B) An ab^{26} mutant dbd neuron. The arrow indicates the ectopic dendritic processes. (C) A wild-type ddaE neuron extends smooth dendrites toward the posterior segment boundary. (D) An ab^{26} mutant ddaE neuron with increased terminal dendritic branches. (E) Quantification of dendritic ends in wild-type or ab^{26} mutant ddaE neurons. (F) A wild-type ddaF neuron. (G) An ab^{26} mutant ddaF neuron with increased terminal dendritic branches. (H) Quantification of dendritic ends in wild-type or *ab* mutant ddaF neurons. Values are mean ± SEM. For MARCM analysis, single neurons in A1 to A7 segments were recorded. *$p < 0.05$. Scale bar, 50 μm.

Because ab^{26}/ab^{26} is embryonic lethal, we analyzed the phenotype of ddaE neurons in *ab* mutant larvae in which ab^{26} was used in heteroallelic combination with a weak adult viable allele ab^{1} (Hu et al., 1995; Cook et al., 2004), so that the ab^{26}/ab^{1} mutants were viable and we could analyze dendritic phenotype in the third instar stage. Ab^{1} homozygous mutants did not have a detectable change in the level of Ab expression in larval DA neurons and $ab^{1}/+$ larvae did not exhibit any dendritic phenotype (data not shown). In these ab^{26}/ab^{1} mutant larvae, ddaE neurons in A5 segments had 19% more dendritic branches than the wild-type. We found that dendritic phenotype in ab^{26}/ab^{1} ddaE neurons could be rescued by expressing *UAS-ab* in ddaE neurons using the neuronal subtype-specific line $Gal4^{221}$ that drives target gene expression in ddaE, ddaF, and vpda neurons (Grueber et al., 2003) (Figure 4A). The notion that Ab controls dendritic branching of a subset of MD sensory neurons is further supported by the detection of Ab expression in the nuclei of ddaE, ddaF, and dbd neurons, but not

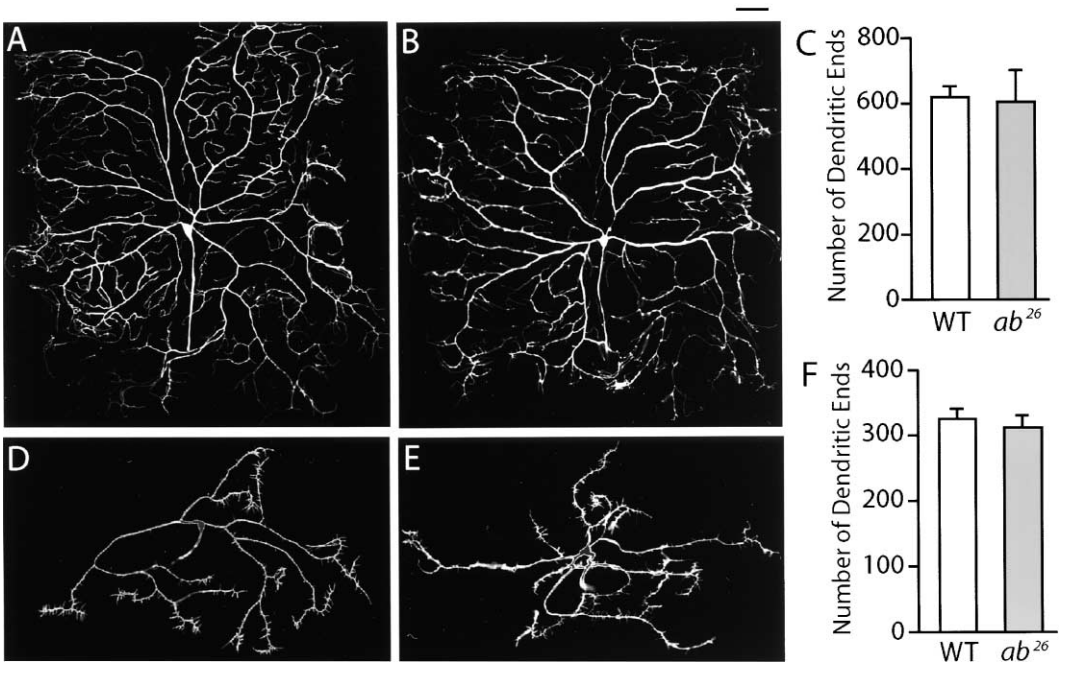

Figure 3. The ab^{26} Mutation Does Not Affect MD Neurons with Complex Dendritic Branching Patterns as Shown by MARCM

(A) Dendritic branching pattern of a wild-type ddaC neuron. (B) Dendritic branching pattern of an *ab* mutant ddaC neuron. (C) Quantification of dendritic ends in wild-type (WT) and *ab* mutant ddaC neurons. (D) Dendritic branching pattern of a wild-type ddaA neuron. (E) Dendritic branching pattern of an *ab* mutant ddaA neuron. (F) Quantification of dendritic ends in wild-type and *ab* mutant ddaA neurons. There was no statistically significant difference between wild-type and *ab* mutants in either case. Values are mean ± SEM. Scale bar, 50 μm.

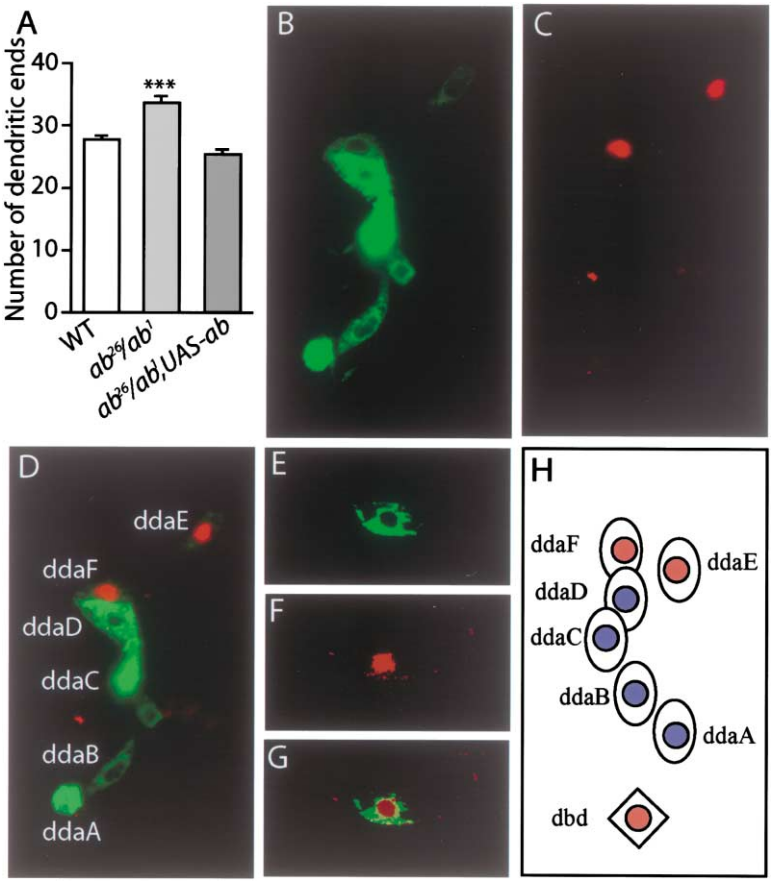

Figure 4. Ab Is Expressed in dbd and a Subset of DA Neurons in the Dorsal Cluster that Do Not Express Cut

(A) Genetic rescue of dendritic phenotype in *ab* mutants. The number of dendritic ends of ddaE neurons increased in ab^{26}/ab^{1} mutants, which could be rescued by expression of *UAS-ab* in these neurons with driver $Gal4^{221}$. ***$p < 0.001$.

(B) GFP labeling of all MD neurons in the dorsal cluster using driver *Gal4109(2)80*.

(C) Immunostaining of Ab expression in dorsal cluster sensory neurons.

(D) Merged image of (B) and (C) to indicate that Ab is only expressed in two of the six DA neurons.

(E) GFP labeling of the dbd neuron.

(F) Immunostaining of Ab expression in a dbd neuron.

(G) Merged image of (E) and (F).

(H) A cartoon indicating the distinct but complementary expression of Ab (red) and Cut in (blue) dbd and dorsal cluster DA neurons. The tracheal innervating neuron in the dorsal cluster extends only a single dendrite and do not express Ab or Cut therefore is not represented here.

in other DA neurons at larval stages (Figures 4B–4G) and in embryos (data not shown). The Ab expression pattern correlated well with the finding that only a subset of neurons were affected by *ab* mutations. The Ab-positive DA and dbd neurons in the dorsal cluster are always Cut negative, and vice versa (data summarized in cartoon in Figure 4H). Taken together, these studies indicate that Ab is a neuronal subtype-specific intrinsic regulator of dendritic branching.

Ab Suppresses Dendritic Branching Independently of Cut

Cut, a homeodomain transcription factor, is expressed at a low level in ddaB neurons (class II), a higher level in ddaC neurons (class IV), and at the highest level in ddaA neurons (class III). Loss of *cut* activity dramatically decreased dendritic growth and branching of all the Cut-positive sensory neurons (Grueber et al., 2003). To test whether Ab can suppress dendritic branching of Ab-negative, Cut-positive DA neurons, we first ectopically expressed Ab in all DA neurons with the driver *Gal4109(2)80*. This caused a 40% reduction in the number of dendritic ends of all dorsal cluster DA neurons in second instar larvae ($n = 9$ for each genotype, $p < 0.01$). To analyze this phenotype in detail, we examined all three classes of DA neurons that were Ab negative, Cut positive. We first expressed Ab in ddaC neurons using neuronal subtype-specific $Gal4^{477}$ that drives target gene expression in class IV neurons only (Grueber et al., 2003). Dendritic branching was reduced by 51% due to the ectopic expression of Ab (Figures 5A–5C). Due to the lack of specific drivers for class II and III DA neurons, we used *Gal4109(2)80* to ectopically express Ab and traced the dendritic trees of vdaA neurons (class II) and ldaB neurons (class III) in second instar larvae. We found a similar decrease in the number of dendritic ends on vdaA neurons (59%, $n = 10$, $p < 0.001$) and ldaB neurons (51%, $n = 10$, $p < 0.001$).

Since Ab is expressed in dbd, ddaE, and ddaF neurons, which do not express Cut, we wondered whether Ab functions by inhibiting *cut* activity. Cut expression was not altered in *ab* mutant embryos, and no Cut was detected in ddaE, ddaF, or dbd neurons (Figures 5D and 5E). Conversely, ectopic expression of Ab in normally Ab-negative, Cut-positive neurons did not affect the expression of Cut (Figure 5F). These findings suggest that Ab controls dendritic branching of a subset of MD neurons through a transcriptional program that does not require Cut.

To further explore how the transcription programs controlled by Ab and Cut exert their effects, we also examined the Ab expression in *cut* mutant neurons. We generated *cut* mutant single-neuron clones in the dorsal cluster using the MARCM technique and found reduced dendritic branching as reported before (Grueber et al., 2003). We stained the mutant neurons with anti-Ab antibody and found that Ab was not upregulated in *cut* mutant MD neurons ($n = 5$), while Ab was still detected

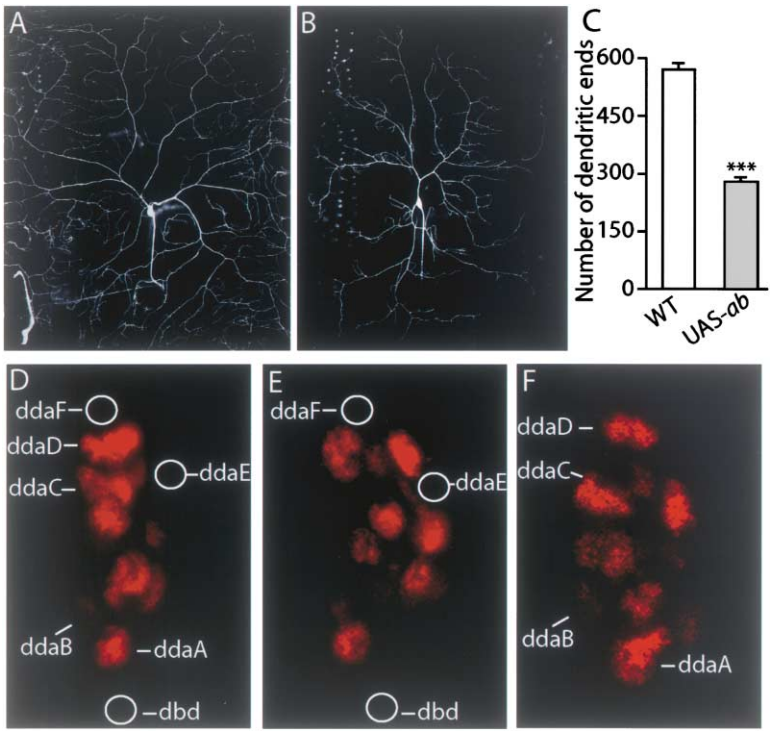

Figure 5. Ectopic Expression of Ab Suppresses Dendritic Branching

(A) A wild-type ddaC neuron that normally does not express Ab.

(B) Ectopic expression of Ab in ddaC neurons with neuronal subtype-specific *Gal4*^{477} reduces dendritic branching.

(C) Statistical analysis of the effect of ectopic expression of Ab in ddaC neurons. Twenty neurons were recorded for each genotype (***$p < 0.001$).

(D) Cut expression pattern in wild-type embryos.

(E) Cut expression is not affected in *ab*^{26} mutant embryos. Note that Cut was undetectable in ddaE, ddaF, and dbd neurons.

(F) Ectopic expression of Ab in normally Ab-negative, Cut-positive neurons in embryos under the control of driver *Gal4 109(2)80* did not affect the expression of Cut.

in adjacent Ab-positive neurons (Figures 6A–6C). This finding suggests that, in wild-type Ab-negative, Cut-positive neurons, the absence of Ab expression is not due to the suppression by Cut, further supporting the notion that Ab and Cut control two transcription programs that are independent of each other. Interestingly, when Cut was ectopically expressed in wild-type Ab-positive, Cut-negative MD neurons, the expression of Ab was suppressed (Figures 6D and 6E), suggesting that Cut is capable of suppressing Ab expression under artificial circumstances. To investigate whether the Ab and Cut transcription programs control some overlapping downstream pathways in different subsets of neurons, we coexpressed Ab and Cut in Ab-positive, Cut-negative MD neurons. Ectopic expression of Cut dramatically increased the dendritic growth and branching of these neurons (Figures 6F and 6G and Grueber et al., 2003). Although ectopic expression of Cut suppresses the expression of endogenous Ab, coexpression of Ab in Ab-positive, Cut-negative neurons did not rescue the phenotype of increased dendritic branching caused by Cut (174.5 ± 7.8, $n = 12$ versus 166.3 ± 8.7, $n = 12$; $p > 0.4$) (Figures 6H and 6I), again suggesting that Cut promotes dendritic branching without suppressing Ab. However, coexpression of Ab partially rescued the phenotypes of increased total dendritic length (3.15 ± 0.09, $n = 12$ versus 4.57 ± 0.15 mm, $n = 12$; $p < 0.001$) (Figure 6J) and dendritic field size (5.70 ± 0.27, $n = 12$ versus $9.81 \pm 0.44 \times 10^4$ μm^2, $n = 12$; $p < 0.001$) caused by Cut (Figure 6K). Overexpression of Ab alone in Ab-positive, Cut-negative neurons did not significantly affect total dendritic length or dendritic field size (data not shown and please also see Figure 7). These findings suggest that Ab and Cut control overlapping downstream pathways that regulate dendritic growth.

Ab Functions in a Dosage-Dependent Manner

Next, we determined whether the effects of Ab on dendritic branching of a subset of PNS sensory neurons were dosage dependent. *Ab*^{26} homozygous mutants are embryonic lethal, and the missense mutation in the BTB/POZ domain likely makes the protein functionally null. In *ab*^{26}/+ heterozygous third instar larvae, the number of dendritic ends of ddaE neurons in the A5 segment was significantly higher than in wild-type larvae (32.8 ± 0.8, $n = 30$ versus 27.9 ± 0.6, $n = 30$, $p < 0.001$) (Figures 7A–7C). This dendritic phenotype in *ab* heterozygous larvae is less severe than *ab* homozygous mutant neurons (Figure 2). In this analysis, all the dendrites of ddaE neurons were counted that were labeled by GFP under the control of *Gal4*^{221}. *Df(2L)FCK-20*/+ larvae also exhibited a similar phenotype (Figure 7C), providing additional genetic evidence for the dosage-dependent effect of *ab* on dendritic branching.

We also examined vpda neurons that express Ab and are located in the ventral cluster (Figure 7D, inset). The dendritic trees of vpda neurons can be easily traced when all MD neurons are labeled by GFP under the control of *Gal4 109(2)80* or *Gal4*^{221}. Compared with ddaE neurons, the dendritic branching phenotype of vpda neurons caused by partial reduction in *ab* activity was more pronounced. *Ab*^{26}/+ heterozygous larvae had significantly more dendritic ends than wild-type larvae (55.9 ± 1.3 versus 40.7 ± 0.6, $n = 30$/group, $p < 0.001$) (Figures 7D–7F). Again, a similar dosage-dependent effect on dendritic branching was observed in *Df(2L)FCK-20*/+ heterozygous larvae (Figure 7F) as well as in *ab*^{KG05262}/+ or *ab*^{02807}/+ heterozygous larvae (data not shown). The dendritic phenotype of vpda neurons could also be rescued by expression of the wild-type *ab* gene using the neuronal subtype-specific driver *Gal4*^{221} (data

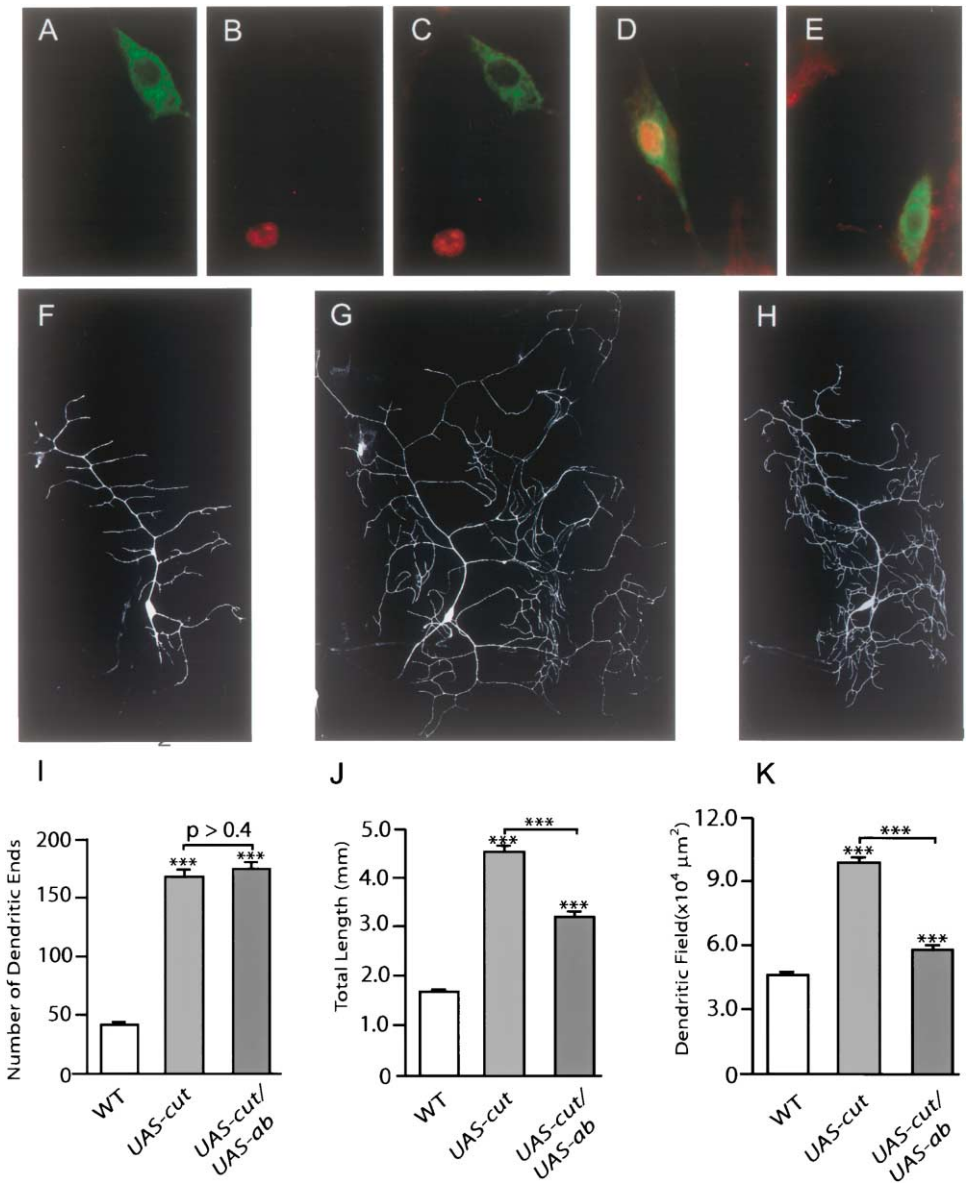

Figure 6. Interactions between Downstream Pathways of Ab and Cut

(A) A *cut* mutant ddaC neuron labeled by GFP.

(B) The same larvae was dissected and stained with anti-Ab antibody. Note that one Ab-positive neuron is present in the field.

(C) A merged image between (A) and (B) to show the absence of Ab staining in *cut* mutant neuron.

(D) A wild-type Ab-positive, Cut-negative neuron labeled by GFP.

(E) Cut overexpression in a normally Ab-positive, Cut-negative neuron suppressed Ab expression.

(F) A wild-type vpda neuron.

(G) A vpda neuron overexpressing Cut.

(H) A vpda neuron overexpressing both Cut and Ab.

(I) Statistic analysis of the number of dendritic ends.

(J) Statistical analysis of the total dendritic length.

(K) Statistical analysis of the size of the dendritic field. ***$p < 0.001$.

not shown). We also measured the total dendritic length, which did not show a statistically significant change in heterozygous larvae (data not shown). This is probably due to the fact that most extra branches are higher-order short dendrites (please see below). The presence of the same dendritic branching phenotype in heterozygous larvae with different *ab* alleles or deficiency indicates that *ab* functions in a dosage-dependent manner.

Further supporting this notion, overexpression of *ab* in ddaE and vpda neurons with the neuronal subtype-specific driver *Gal4*^{221} decreased dendritic branching of these neurons (Figures 7C and 7F). The less severe overexpression phenotype in ddaE and vpda neurons than in Ab-negative, Cut-positive neurons (such as ddaC) is probably due to the high level of Ab already present in Ab-positive, Cut-negative neurons. Taken together,

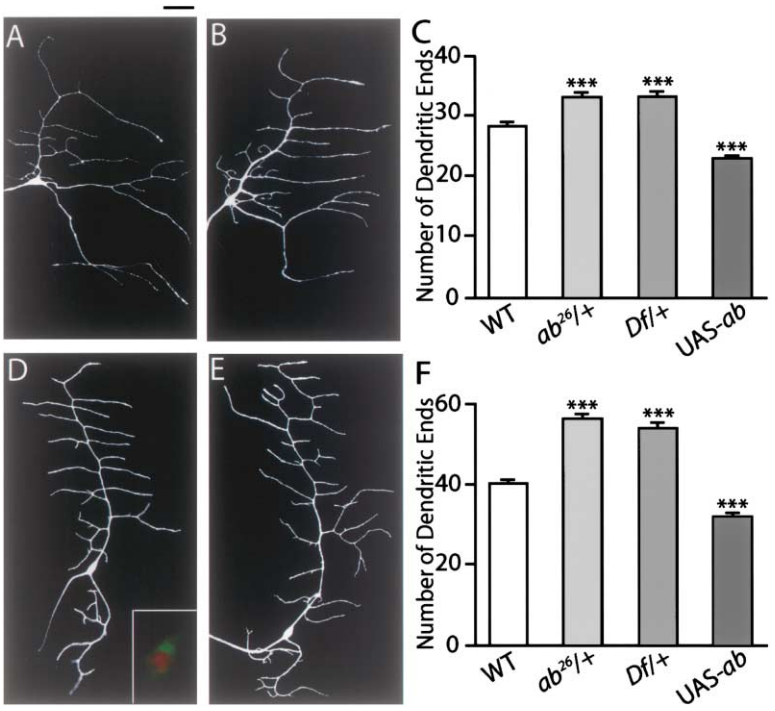

Figure 7. Ab Is a Dose-Dependent Regulator of Dendritic Branching

(A and B) Images of dendritic branching of a ddaE neuron in the A5 segment of wild-type (A) and $ab^{26}/+$ heterozygous (B) third instar larvae. (C) Quantification of the number of dendritic ends of ddaE neurons in wild-type (WT), *ab* heterozygous larvae, and larvae overexpressing Ab using subtype-specific $Gal4^{221}$. (D and E) A vpda neuron in the A5 segment of a wild-type (D) or $ab^{26}/+$ heterozygous (E) third instar larva. The inset in (D) shows the expression of Ab (red) in a vpda neuron (green). (F) Quantification of the number of dendritic ends of vpda neurons in wild-type, *ab* heterozygous larvae and larvae overexpressing Ab using neuronal subtype-specific $Gal4^{221}$. ***$p < 0.001$ by Student's t test. Values are mean ± SEM. In this figure, only neurons in the A5 segment were recorded. Scale bar, 50 μm.

these findings indicate that the level of Ab activity in a neuron directly regulates the complexity of its dendritic branching.

Ab Primarily Affects the Formation of Higher-Order Dendritic Branches

More detailed analysis of dendritic trees indicated that *ab* primarily controls the formation of higher-order dendritic processes of vpda neurons (Figure 8A). In $ab^{26}/+$ heterozygous larvae, the number of primary dendritic branches was identical to that in wild-type larvae; however, the tertiary and quaternary branches were much more severely affected (Figure 8A). Consistent with our MARCM analysis (Figure 3), deleting one copy of *ab* did not affect MD neurons with more complex dendritic morphology, such as ddaC neurons and v′pda neurons, which are located in the ventral cluster and exhibit numerous spine-like fine structures on their dendritic shafts (not shown). To determine whether Ab may also have a function in mature neurons in late stages, we compared the dendritic branching complexity of vpda neurons in second and third instar larvae. Second instar *ab* heterozygous larvae showed only a 12% increase in the number of dendritic ends of vpda neurons as compared to wild-type. However, in third instar larvae, vpda neurons exhibited 39% more dendritic ends in ab^{26}

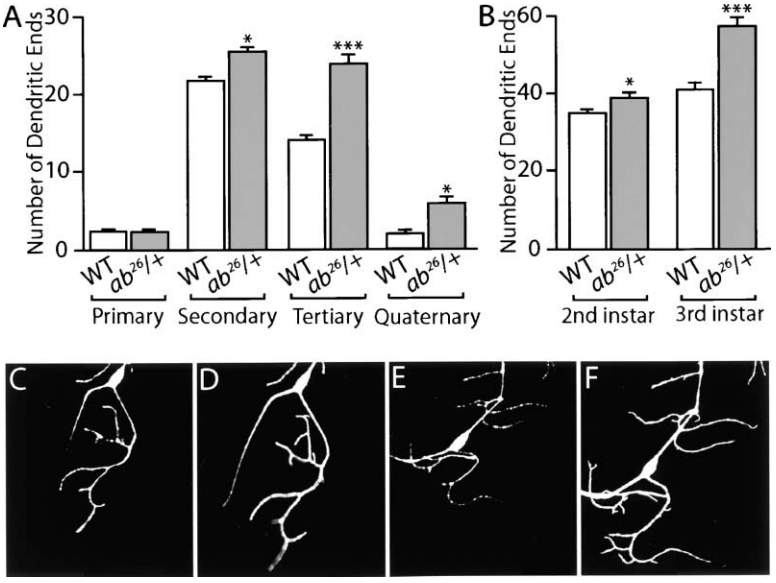

Figure 8. Ab Primarily Affects Higher-Order Dendritic Branches and also Has a Function in Mature Neurons

(A) The number of different orders of dendrites of vpda neurons in wild-type and ab^{26} heterozygous larvae. Branch ordering schemes were generated using the centrifugal method (Uylings et al., 1975). (B) Statistic analysis of *ab* function at late developmental stages. (C) Part of vpda neuron dendritic arbors in a wild-type second instar larva. (D) The same vpda neuron in panel (C) at the third instar larval stage. (E) Part of vpda neuron dendritic arbors in an $ab^{26}/+$ second instar larva. (F) The same vpda neuron in panel (E) at the third instar larval stage. *$p < 0.05$; ***$p < 0.001$, by Student's t test. Values are mean ± SEM.

heterozygous larvae compared to wild-type controls (Figures 8B–8F). This finding, together with the fact that Ab expression persists in third instar larvae, suggests that Ab not only suppresses dendritic branching during embryogenesis but also may continue to function in more mature neurons at late developmental stages.

Discussion

Here we report that Ab functions cell-autonomously in a subset of postmitotic neurons to limit dendritic branching. Ab does so in a transcriptional program that does not require Cut. More importantly, the level of Ab activity directly correlated with dendritic complexity of a specific neuron, suggesting that modulating Ab activity in these neurons could be an effective means to fine tune dendritic branching complexity during development.

Ab Cell-Autonomous Function in Dendritic Morphogenesis

The BTB/POZ protein Ab was first identified as an important regulator that controls the specificity of neuromuscular connections between a subset of motoneurons and a subset of muscles (Hu et al., 1995). Interestingly, Ab is expressed in the nucleus of muscle cells but not motoneurons, indicating that it affects the targeting of motoneuron axon terminals in a non-cell-autonomous fashion. It remains unknown what downstream targets are misregulated in muscle cells in *ab* mutants that are responsible for mediating the interactions between motoneuron axon terminals and the muscle surface. Here we provide evidence that Ab has a cell-autonomous function in neural development.

Ab is expressed in the nucleus of a subset of postmitotic MD sensory neurons (Figure 4). *Ab* mutant embryos have a normal number of MD neurons that can still be labeled by a pan-MD marker *Gal4109(2)80*, suggesting that the *ab* gene does not control the MD fate of these neurons. This notion is in contrast to other transcription factors in *Drosophila* that have a dual function in both cell fate determination and dendritic morphogenesis (Brenman et al., 2001; Grueber et al., 2003; Moore et al., 2002). It is possible that Ab is a transcription factor dedicated to maintaining the less-branched dendritic trees of ddaE, ddaF, and dbd neurons in the dorsal cluster. Here we show that Ab is a transcription factor whose normal function is to limit rather than promote dendritic branching in postmitotic neurons.

MARCM analysis demonstrated that Ab has a cell-autonomous function in postmitotic neurons to directly control dendritic branching during development (Figure 2). The unique features of MD neuron lineages ensure that the presence of a single mCD8-GFP-labeled MD neuron itself indicates that the somatic recombination occurs during the last cell division that gives rise to the MD neuron (Sweeney et al., 2002). Therefore, the dendritic phenotypes we observed in single MD neuron clones reflect the gene function in postmitotic neurons. Although Ab is also expressed in muscle cells and in epidermis, it is unlikely that Ab has a non-cell-autonomous function in controlling dendritic branching of sensory neurons, since expression of *UAS-ab* only in ddaE, ddaF, and vpda neurons in *ab* mutants could rescue the dendritic phenotype.

Transcriptional Code for Controlling Dendritic Branching

In the dorsal cluster, Ab and Cut are expressed in distinct but complementary subsets of DA neurons. Ab is only expressed in ddaE and ddaF neurons in addition to dbd neurons, while Cut is expressed in the four other DA neurons (Figure 4H). (Please note again that ddaF neurons in Grueber et al., 2003, are ddaD neurons in Sweeney et al., 2002, and in this paper.) Ab limits the dendritic branching of neurons with less-branched dendritic trees, while Cut promotes dendritic branching in other neurons with highly branched dendritic trees.

Several lines of evidence indicate that Ab functions in a transcription program that does not require Cut. First, Ab and Cut were expressed in distinct but complementary subsets of neurons in each dorsal cluster. Second, Cut was undetectable in normally Ab-positive, Cut-negative neurons in *ab* mutant embryos, suggesting that Ab does not function by suppressing Cut expression. Third, Cut expression was not affected in Ab-negative, Cut-positive neurons when Ab was ectopically expressed. Taken together, these data show that Ab controls dendritic branching through a transcriptional program that does not require Cut. On the other hand, Ab expression was not detectable even in ddaB neurons where Cut expression was very low and was not upregulated in *cut* mutant neurons that are normally Ab negative and Cut positive. These findings further support the notion that, under normal circumstances, Ab and Cut control two transcription programs independent of each other. Ectopic expression of Cut in Ab-positive, Cut-negative neurons can suppress Ab expression (Figure 6), which is probably due to the fact that Cut and several other transcription factors share common DNA binding sites (Kalionis and O'Farrell, 1993). Actually, Ab itself and Cut could bind to some consensus DNA sequences at least in vitro (Kalionis and O'Farrell, 1993), raising the possibility that the transcription of at least some common target genes is regulated by Ab in one subset of sensory neurons but by Cut in another subset. This notion is further supported by our finding that coexpression of Ab could partially rescue the dendritic overgrowth phenotype caused by ectopic expression of Cut in Ab-positive, Cut-negative neurons (Figure 6).

Our studies reported here provide strong evidence that different transcription factors specifically either promote or inhibit dendritic branching in a neuronal subtype-specific manner. A similar mechanism has been demonstrated in other model systems to control axonal branching. For instance, the zinc finger protein Brakeless controls axon terminal arborization of a subset of photoreceptors in *Drosophila* (Senti et al., 2000). In the spinal cord, the ETS class transcription factor PEA3 regulates axonal branching of specific motoneuron pools (Livet et al., 2002). In the *Drosophila* olfactory system, the POU domain transcription factors Acj6 and Drifter regulate both dendritic targeting specificity and axon terminal arborization (Komiyama et al., 2003). The BTB/POZ domain is known to mediate protein-protein interactions between heterodimers (Bardwell and Treisman,

1994). It is likely that other transcription factors may collaborate with Ab or Cut to provide additional layers of specificity in controlling dendritic branching in a subtype-specific manner.

Dosage-Dependent Effects of Ab on Dendritic Branching

An important finding of this study is the dosage-dependent effect of Ab on dendritic branching in a given neuron. Ab-positive, Cut-negative neurons, but not Ab-negative, Cut-positive neurons, exhibit increased dendritic branching in *ab* heterozygous larvae or *Df*/+ larvae, while overexpression of Ab resulted in decreased dendritic branching in Ab-positive, Cut-negative neurons (Figures 7 and 8). These findings suggest that dendritic branching complexity is tightly regulated at the transcriptional level and that Ab is a key component in this regulatory pathway. The evolutionarily conserved BTB/POZ domain can promote transcriptional repression by recruiting corepressor proteins (Dhordain et al., 1997; Hong et al., 1997). Different levels of Ab may form qualitatively or quantitatively different complexes that in turn regulate the expression level of its target genes. Fine regulation of Ab availability or activity might be an effective way to control the dendritic branching complexity of a specific neuron in response to neuronal activity or different environmental stimuli.

Experimental Procedures

Fly Stocks

All the genetic crosses were carried out at 25°C with standard food medium. The following stocks were used in this study: (1) *Gal4 109(2)80, UAS-GFP* (Gao et al., 1999); (2) *Gal4 109(2)80, UAS-mCD8::GFP*; (3) *CyO*, *Krüppel-Gal4, UAS-GFP* (kindly provided by T. Kornberg); (4) *Gal4 109(2)80*, *UAS-GFP*, ab^{26}/*CyO*, *Krüppel-Gal4*, *UAS-GFP* (Gao et al., 1999); (5) *UAS-ab;* ab^{1} (Cook et al., 2004). Ab^{1} is an adult viable allele, and the mutation was thought to be in the regulatory region that bypasses early embryonic requirement for this gene and only affects wing vein development in adults (Hu et al., 1995; Cook et al., 2004). (6) $Gal4^{221}$, UAS-*mCD8::GFP, UAS-cuta, w* cut^{c145} FRT^{19A}/*y*+*ct*+*Y*, and $Gal4^{477}$, UAS-*mCD8::GFP* $Gal4^{C155}$ (Grueber et al., 2003); (7) *UAS-mCD8::GFP*, *hs-FLP1*; (8) *tubP-Gal80*, FRT^{40A}/*CyO*; (9) *KG05262/CyO*, *l(2)K02807/CyO*, and ab^{1} (from the Bloomington Stock Center).

Mapping

The second chromosome deficiency kit (DK-2, Bloomington Stock Center) was used to identify deficiency lines that cover the lethal mutations in ab^{26} induced by ethyl methanesulfonate. We then used a number of P element insertion lines or small deletion lines (Bloomington Stock Center) to further narrow down the locus of the mutation in *ab*. To identify *ab* mutations, we isolated genomic DNA from homozygous ab^{26} mutant embryos scored by the absence of the *Krüppel-Gal4 GFP* balancer and the presence of the homozygous mutant GFP chromosome. Primers were designed based on the wild-type gene sequence from FlyBase (http://flybase.bio.indiana.edu), and independent polymerase chain reactions were carried out to clone and sequence both strands of the mutant DNA.

Quantitative Analysis of Dorsal Cluster DA Neuron Dendrites

Staged wild-type or *ab* mutant embryos were collected on grape agar plates and processed as described (Gao et al., 1999). In brief, embryos were collected and kept at 25°C overnight, dechorionated with 2.5% sodium hypochlorite, and mounted in 90% glycerol in phosphate-buffered saline (PBS). The dendritic morphology of GFP-labeled dorsal DA neurons was recorded by a confocal microscope (Nikon, D-Eclipse C1), and the total number of dendritic branches in the dorsal cluster was counted. For the *ab* dose-dependence study, wild-type or *ab* heterozygous embryos were collected and kept at 25°C for 4 days. Third instar larvae were collected, and images of GFP-labeled dorsal and ventral cluster DA neurons were obtained as described above. Because DA neurons elaborate their dendrites underneath the epidermis, a single image was taken for each neuron that encompassed the entire dendritic arbor. All the dendritic ends were counted and analyzed to reflect the branching complexity. In some cases, different orders of dendritic branches were counted as well using the centrifugal method (Uylings et al., 1975). According to this method, branches that extend directly from cell body are defined as primary dendrites, and branches that extend from primary dendrites are defined as secondary dendrites, and so on. The total length of dendritic branches was calculated on a computer. The dendritic field area was defined as the smallest bounding convex area that could circumscribe the entire dendritic field. All statistical analyses were done using Student's t tests.

Single-Neuron MARCM

The cell-autonomous function of *ab* in single DA neurons was analyzed as described (Li and Gao, 2003). First, the ab^{26} mutation was recombined onto the chromosome containing FRT^{40A}. $ab^{26, FRT40A}$/*CyO* male flies were crossed with $Gal4^{C155}$, *UAS-mCD8::GFP*, *hs-FLP1/FM7* virgin flies. Then, $Gal4^{C155}$, *UAS-mCD8::GFP*, *hs-FLP1*; $ab^{26, FRT40A}$/+ male flies were crossed with $Gal4^{C155}$, *UAS-mCD8::GFP*, *hs-FLP1*; *tubP-Gal80,* FRT^{40A}/*CyO* virgin flies. For *cut* MARCM analysis, *w* cut^{c145} FRT^{19A}/*y*+*ct*+*Y* and *tubP-Gal80*, FRT^{19A}/*FM7*, *Gal4 109(2)80*, *UAS-GFP* line were used. Embryos from these crosses were collected on grape agar plates for 3 hr in a 25°C incubator. The embryos were aged for 3 hr and then heat-shocked in a 37°C water bath for 40 min to induce mitotic recombination. The embryos were then kept in a moisture chamber at 25°C and allowed to develop for 3–4 days. Third instar larvae were collected, and those larvae that contained a single mCD8::GFP-labeled dorsal cluster PNS neuron were selected under a Nikon fluorescence dissection microscope. Images of dendritic morphology of single DA neurons were recorded with a Nikon confocal microscope (D-Eclipse C1). The significance of difference in dendritic branching complexity was determined with Student's t tests.

Immunohistochemistry

For antibody immunostaining of DA neurons in embryos or dissected third instar larvae, rabbit polyclonal antibody against Ab (1:500 dilution, Cook et al., 2004) or mouse anti-Cut antibody (1:20 dilution, Developmental Studies Hybridoma Bank) was used as the primary antibody. Cy3-conjugated goat anti-rabbit or anti-mouse IgG (Jackson ImmunoResearch Laboratories, 1:100) was used as the secondary antibody. The embryos or dissected larvae were mounted in 90% glycerol in PBS, and confocal images were obtained with a confocal microscope (Nikon, D-Eclipse C1).

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